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Induction of Metamorphosis Causes Differences in Sex-Specific Allocation Patterns in Axolotls (*Ambystoma mexicanum*) that Have Different Growth Histories

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Abstract

We tested the hypothesis that salamanders growing at different rates would have allocation patterns that differ among male and female metamorphic and larval salamanders. We raised individual axolotls, *Ambystoma mexicanum*, on four food regimes: constant high growth (throughout the experiment), constant low growth (restricted throughout the experiment), high growth switched to low growth (ad libitum switched after 140 d to restricted), and low growth switched to high growth (restricted switched after 140 d to ad libitum). Because axolotls are obligate paedomorphs, we exposed half of the salamanders to thyroid hormone to induce metamorphosis. We assayed growth and dissected and weighed gonads and fat bodies. Salamanders that were switched from restricted to ad libitum food regime delayed metamorphosis. In all treatment groups, females had larger gonads than males and males had larger fat bodies than females. The association between storage and reproduction differed between larvae and metamorphs and depended on sex.

Life histories of most amphibians are more complex than those of other tetrapods because many species of amphibians undergo metamorphosis. Nutritional energy may be allocated to several other competing processes (e.g., storage, maturation, growth) that can affect lifetime reproductive potential (Dunham et al., 1989; Bernardo, 1994). Therefore, metamorphic timing or size may not necessarily be optimized in ways predicted by theory (Wilbur and Collins, 1973; Werner, 1986; Alford, 1999; Harris, 1999; Day and Rowe, 2002). For example, there is significant within-species geographic variation in metamorphic age and size in the desmognathan salamanders (e.g., Bruce, 1972, 1988; Camp et al., 2000), but demographic comparisons and theoretical considerations indicate that age at maturation or egg size are likely to be the primary targets of selection rather than metamorphosis (Tilley, 1980; Bernardo, 1994; Beachy, 1995; Beachy and Bruce, 2003). Therefore, variation in metamorphic size or timing or both may simply be a correlate of these variables, i.e., egg size and maturation age (Bernardo, 1994; Beachy, 1995). These allocation variables are correlated because the endocrine system can provide for physiological linkages among these

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variables (e.g., Moriya, 1983; Wakahara, 1994; Kenki and Wakahara, 1999). This can result in allocation tradeoffs that prevent understanding of single traits when studied alone.

In addition to the complex allocation responses that metamorphic salamanders can exhibit, growth can have significant effects on life-cycle expression by influencing the differential expression of paedomorphosis and metamorphosis in facultatively paedomorphic salamanders (Eagleson, 1976; Denoël and Joly, 2000; Ryan and Semlitsch, 2003). Furthermore, Whiteman (1997) suggested that facultatively paedomorphic salamanders have sex-specific differences in fitness that result in between-sex variation in expression of paedomorphosis. Females have larger commitment to gonads than males because eggs are energetically more demanding to produce (e.g., they contain yolk), and male salamanders generally mate at earlier ages (e.g., Tilley, 1980; Bruce, 1988; Whiteman, 1997; Ryan and Bruce, 2000; Beachy and Bruce, 2003). This sex difference, when combined with the influence of growth on development, means that the sex-specific expression of paedomorphosis is complex (Denoël et al., 2005). For example, the paedomorphic part of the population can either be male-biased (Whiteman, 1997; Winne and Ryan, 2001; Whiteman et al., 2012) or female-biased (Denoël, 2003).

We tested the hypothesis that variation in larval growth history causes variation in allocation patterns in salamanders. We then explored how these allocation patterns varied 1) between larval and metamorphic salamanders and 2) between males and females. We grew individual larvae of the obligately paedomorphic salamander *Ambystoma mexicanum* (axolotl), then assayed four components of life history allocation: metamorphic timing (in axolotls treated with thyroid hormone), storage (fat body mass), reproduction (gonad mass), and metamorphic/final size.

We grew individual axolotls at four different rates: constant high growth (HH), constant low growth (LL), high growth switched to low growth (HL), and low growth switched to high growth (LH). We induced metamorphosis in half of the axolotls and then euthanized all animals to determine sex and assay allocation to storage and reproduction. Using an obligate paedomorph allowed us to control the initiation of metamorphosis.

Materials and Methods

We obtained embryos from the *Ambystoma* Genetic Stock Center (University of Kentucky) in March 2007. To reduce genetic variation, we used salamanders from a single cross (e.g., Alford and Harris, 1988). We placed 60 eggs in five 28 × 15 × 10-cm plastic boxes filled with 3.7 L of reverse osmosis (RO) water. Upon hatching (March 15), 56 larvae were placed individually into plastic boxes. Every day, larvae were removed, and the boxes were cleaned and refilled with fresh RO water. Larvae were replaced into the boxes and then were provided 25 mL aliquots of newly hatched brine shrimp. At day 30 (April 14), we increased the brine shrimp to 50-mL aliquots. After day 38 (April 22), larvae were fed California blackworms (*Lumbriculus ordinoides*). After this date, we changed their water twice per week rather than daily. At day 116 (July 9), we transferred the larvae to larger individual plastic containers (35 × 27 × 15 cm) filled with 9 L of RO water.

At day 39 (April 23), we initiated food manipulation via four treatments: HH, HL, LH, and LL (Table 1). HH larvae had worms available to eat at all times. “Restricted” was defined as larvae being fed only twice weekly. In restricted treatments, all worms were consumed within 24 h after feeding. The switch in food availability for HL and LH larvae occurred at day 140. On day 205, we manipulated development by inducing metamorphosis by immersion in 5-nM T₄ (thyroxine) or retaining the larval status by immersion in a control solution. Both treatments were crossed in a full factorial design (4 × 2) for eight treatment groups. This design was replicated in seven spatial blocks for a total of 56 larvae (Table 1).

We weighed larvae at day 115, day 140, day 170, and day 204 to verify intended growth differences. At each weighing, every larva was blotted dry, weighed to the nearest milligram, and returned to its respective box. Upon metamorphosis, salamanders were again weighed, duration of larval period was recorded (as days of thyroid hormone treatment), and the salamander was preserved, along with its sister control treatment larva (see below).

Thyroid hormone treatment was via a T₄ aliquot that brought the animal container solution to 5 nM T₄. Because T₄ is soluble only in basic solution, we added an equally sized aliquot of basic solution (KOH) minus T₄ to all control treatments. Changes in pH in the water were nondetectable after addition of the T₄ aliquot. T₄ and control aliquots were added at every water change, and at the same time the larvae were fed according to treatment group.

Metamorphosis was considered complete with reabsorption of gill fimbriae and tail fin. Date of metamorphosis and metamorphic mass were recorded. Metamorphs were killed in 3% solution of MS-222, fixed for 7 d in 10% formalin, and stored in 70% ethanol. In addition, as each metamorph was preserved, a sister control (i.e., larval) salamander from the same spatial block also was weighed, killed, fixed, and stored to provide a time-controlled comparison of allocation between metamorphs and larvae. After storage of all salamanders, individuals were dissected and the right gonad and fat body were removed and weighed to the nearest milligram.

To confirm expected growth effects, we visually inspected growth profiles, and compared salamander masses at each of the four measurement dates with the use of a one-way ANOVA at each measurement date. After confirming variation in growth history (Fig. 1) we conducted a series of analyses. First, we analyzed the vector responses (consisting of body mass [final mass for larvae and metamorphic mass for metamorphs], gonad mass, and fat body mass) of all salamanders in a two-factor (i.e., growth and T₄ treatments) MANOVA (on the full vector) and three two-factor ANOVAs (one for each life-history trait). Next, we conducted ANOVAs on the separate responses of larval and metamorphic salamanders. We carried out 1) three one-factor ANOVAs examining the effect of growth treatment on final mass, gonad mass, and fat body mass of larvae and 2) four one-factor ANOVAs examining the effect of growth treatment on larval period, metamorphic mass, gonad mass, and fat body mass of metamorphs.

We then conducted a series of analyses to examine the role of sex-based allocation differences. First, for all salamanders we examined the effect of sex on the allocation vector that included body mass, gonad mass, and fat body mass in a single MANOVA. Second, we

examined the effect of sex on larval period (only for metamorphs), body mass (final mass for larvae and metamorphic mass for metamorphs), gonad mass, and fat body mass. We used three separate series of one-factor ANOVAs for 1) all salamanders, 2) larvae only, and 3) metamorphs only. Finally, with the use of a series of one-factor ANOVAs, we tested the effect of sex in each of the eight combinations of growth treatments and T₄ treatment (i.e., larvae and metamorphs).

Data for metamorphic timing were inverse transformed (Alford and Harris, 1988), and data for metamorphic/final mass, gonad mass, and fat body mass were log-transformed (Sokal and Rohlf, 1995). We used Wilks's λ as our multivariate test statistic ($\alpha = 0.05$). Because we used six preplanned pairwise comparisons, we used a Bonferroni correction to set $\alpha = 0.0083$ as the significance criterion in pairwise LSD comparisons.

Results

Food treatments had their intended effect in producing different larval growth trajectories (Fig. 1). Larvae had different masses at day 115 ($F_{1,54} = 13.4$, $P = 0.001$) and day 140 ($F_{1,54} = 32.3$, $P < 0.001$). After the food switch, growth trajectories of the four food regimes continued to diverge (day 170, $F_{3,52} = 24.4$, $P < 0.001$; day 204, $F_{3,51} = 57.8$, $P < 0.001$). The LH larvae were larger than LL larvae at all time points. The HL larvae were not smaller than HH larvae until day 204. These differences in growth trajectories resulted in differences in the allocation response vectors that consisted of final salamander mass, gonad mass, and fat body mass (Table 2).

The effect of treatment with T₄ was limited to final body mass (Fig. 1), which we expected because of the twofold effect of T₄ causing dehydration and reduced feeding during metamorphosis. Gonad mass and fat body mass did not differ significantly between larvae and metamorphs (Fig. 2). All salamanders were sexually immature.

Significant variation in each variable resulted from growth treatments (Table 2 and Fig. 2). When all salamanders were examined, HH and LH groups grew larger, and had larger gonads and larger fat bodies than LL and HL groups (Fig. 1). We then examined larvae and metamorphs separately and found that HH and LH larvae had larger final sizes and larger fat bodies than LL and HL larvae (Table 3). HH and LH metamorphs had larger final sizes and larger fat bodies than LL and HL metamorphs. In addition, LH metamorphs delayed metamorphosis relative to LL, HH, and HL metamorphs (Table 3, Fig. 2).

Using sex as a single factor in a MANOVA indicated significant differences in allocation vectors between males and females (Wilks's $\lambda = 0.244$; $F_{3,45} = 46.56$; $P < 0.001$). In univariate analyses, all traits except for final body mass differed significantly between the sexes (Table 4). Female mean gonad mass (933.8 mg for metamorphs and 976.3 mg for larvae) was significantly greater than male mean gonad mass (291.3 mg for metamorphs and 308.3 mg for larvae), and male mean fat body mass (240.5 mg for metamorphs and 195.5 mg for larvae) was significantly greater than female mean fat body mass (63.0 mg for metamorphs and 53.0 mg for larvae). Given the low P value for the effect of sex on larval

period, it is tenable that males may metamorphose sooner after T_4 treatment than females (46.5 days vs. 59.8 days in our experiment).

Outside of the effect on final size, T_4 did not affect allocation vectors (Table 2). However, it did affect sex-based differences in allocation (Table 5). The pattern of sex differences in gonad mass varied among food treatments and between larvae and metamorphs (Table 5, Fig. 3). Therefore, we further explored the interaction of growth, T_4 , and sex by conducting an exploratory data analysis wherein we compared the univariate responses of each sex in each treatment group. Even though further dividing treatment groups into another treatment effect (i.e., sex) reduced degrees of freedom and our ability to detect significant differences (e.g., there was only one female in the LH food metamorph treatment group), differences in gonad mass and fat body mass are worth noting.

With regard to gonad mass, two differences were observed. First, larval females had larger gonads than males in all food treatments except for the LL treatment, whereas metamorphic females had larger gonads in only LL and HL treatments (Table 5). Second, in LL treatments, T_4 caused a change in allocation leading to an increase in female gonad mass (Fig. 3). In contrast, in LH and HH treatments T_4 caused a change in allocation leading to a decrease in female gonad mass (Fig. 3). Male gonad masses were unaffected by T_4 treatment in all growth treatments.

We observed only one difference in fat body mass. LH salamanders exhibited an increase in fat body mass when treated with T_4 (Fig. 3). This increase was more dramatic in males. However, females also shifted allocation to storage when treated with T_4 . These females also shifted allocation away from reproduction, i.e., metamorphic LH females exhibited a tradeoff typically observed in the males. Analyzing males and females separately helped to resolve the marginal significance of food on metamorphic timing. LH females metamorphosed later ($F_{3,6} = 9.08$, $P = 0.012$) but did not have larger fat bodies than all other females ($F_{3,6} = 1.78$, $P = 0.251$). When LH males are compared to other males, no significant variation in metamorphic timing is apparent ($F_{3,8} = 0.396$, $P = 0.76$) despite possessing larger fat bodies ($F_{3,8} = 8.36$, $P = 0.008$).

Discussion

We tested the hypothesis that sex-specific differences in allocation are influenced by growth history and metamorphosis. We intended to explore how growth history influenced the expression of alternate life cycles (metamorphic vs. larval), how growth history affects metamorphic timing, and how the resultant variation in metamorphic/larval state was linked to variation in allocation to other essential features of the salamander life history, i.e., storage and reproduction.

Growth history affected allocation patterns differently. We found differences in reproduction (gonad mass) and storage (fat body mass) but not in overall mass or metamorphic timing (larval period). The most obvious pattern was that salamanders grown in a consistently good environment were larger than those grown in a consistently bad environment. In real ponds, growth varies for several reasons, e.g., density, presence of predators, food availability,

temperature, and desiccation (Scott, 1990; Newman, 1998; Relyea, 2002). The influence of these factors on metamorphosis is complex (Wilbur and Collins, 1973; Alford and Harris, 1988; Day and Rowe, 2002). Our results indicated that salamanders that experience poor growth initially but have rapid growth later in the larval period will delay metamorphosis. This could be a mechanism for taking advantage of a positive growth environment (Wilbur and Collins, 1973). The other growth groups did not vary significantly in age at metamorphosis. Interestingly, the larvae that delayed metamorphosis also exhibited greater storage. This suggests a potential endocrinological linkage between metamorphosis and storage. It seems tenable that larval amphibians may use storage rather than growth as a modulator of metamorphosis with the adaptive significance of continuing to grow in a productive environment. HH larvae metamorphosed earlier than LH larvae but at a similar size. This supports the notion of a maximal size threshold that is indicated by several models (Wilbur and Collins, 1973; Werner, 1986; Rowe and Ludwig, 1991).

Although we found many allocation differences, the clearest were those between males and females. Males allocated more to storage than reproduction, whereas females had larger gonads at the cost of storage. Although this is not an unusual observation, it also may account for the longer larval period of females than males in our experiment. In some surveys, male *Ambystoma* are more often paedomorphic than females (Whiteman, 1997; Winne and Ryan, 2001), and females that are paedomorphic appear to accelerate maturation as a trade-off to growth, i.e., female paedomorphic *Ambystoma talpoideum* are smaller than immature female larval *A. talpoideum* (Winne and Ryan, 2001). Female paedomorphic *A. talpoideum* have a higher metabolic rate than males (Ryan and Hopkins, 2000), and this elevated metabolism seems to be associated with the high demand for generating ova. The opposite pattern also has been seen, e.g., paedomorphic *Ambystoma mavortium* from North Dakota are female-biased (Poitra et al., 2007). These populations can experience faster growth and achieve larger larval and paedomorphic sizes than the *Ambystoma* described above. In our experiment, both large females and males (i.e., LH and HH larvae) showed a statistically significant shift in allocation from reproduction to storage when induced to metamorphose, and this may be associated with the shorter larval period in males when compared to females in our experiment. Perhaps sex-based differences in larval period can be explained by the growth environment that larvae experience.

We also found sex-based differences when comparing the individual LH female with the rest of the females. This female delayed metamorphosis and also shifted allocation from reproduction to storage when induced to metamorphose. Interestingly, we also saw this response consistently in males. This “male-like” allocation in the LH female suggests larvae that achieve sufficient energy allocation to reproduction are then capable of a significant shift to storage in a good growth environment.

Ryan and Semlitsch (2003) postulated that growth opportunities at particular points in the developmental life cycle of a salamander are more important than the overall developmental life cycle. Our results are consistent with this view. In addition, consistent with Whiteman's (1997) results, we found differences in life cycle expression (i.e., days to metamorphosis) between sexes. These differences were associated with size and storage, indicating a potential shift in allocation from reproduction to storage during metamorphosis. Our results

indicate the allocation response of larval salamanders to be as complex as the environment they experience. This means that understanding a single trait (e.g., metamorphic timing) is difficult without concurrent emphasis on other important allocation variables. Furthermore, it is clear the sexes differ in their allocation schedules under all conditions. Gonad mass, fat body mass, and larval period differed between the sexes. Females allocate preferentially to gonad mass to a greater degree than males, with the consequent trade-off for storage. Because larvae may adjust the timing of metamorphosis according to storage potential, this may explain why females delayed metamorphosis compared to males. Allocation to reproduction in females is greater than to other functions (e.g., storage), and this manifests in differential life cycle expression between the sexes. The causes of life-cycle variation (e.g., variation in metamorphic timing) will be difficult to understand without reference to sex-based differences in life cycle expression.

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FIG. 1.

Growth profiles for axolotls grown in four food regimes. Diamonds are LL growth treatments, squares are LH growth treatments, triangles are HL growth treatments, and circles are HH growth treatments. Dashed line indicates the initiation of treatment with thyroxine to induce metamorphosis in half of animals. Open symbols after induction of metamorphosis are growth profiles for larvae (i.e., untreated salamanders). Growth profiles for metamorphs terminate at mean larval period. Error bars are ± 1 SE.

FIG. 2.

Treatment-group means of four allocation variables for larval (i.e., untreated salamanders) and metamorphic (i.e., salamanders treated with TH) axolotls. Because we used six preplanned pairwise comparisons, we calculated a Bonferroni correction to set $\alpha = 0.0083$ as significance criterion in these paired LSD comparisons. Letters next to means indicate means significantly different. Open squares = LL food treatment; open circles = LH food treatment; filled squares = HH food treatment; filled circles = HL food treatment. Error bars are ± 1 SE.

FIG. 3.

Allocation to reproduction (gonad mass) and storage (fat body mass) in male and female axolotls. Closed symbols are larval axolotls, open symbols are metamorphic axolotls (i.e., those treated with TH). Arrows are drawn from larvae to the metamorphs for sex and food regimes. Arrows are included to emphasize the effect of TH treatment on allocation to reproduction and storage. Diamonds are LL growth treatments, squares are LH growth treatments, triangles are HL growth treatments, and circles are HH growth treatments.

TABLE 1

Summary of treatment groups of *Ambystoma mexicanum*. All larvae were initially fed newly hatched brine shrimp. On day 39 of the experiment larvae were fed blackworms either ad libitum or restricted (only as many as could be consumed within 24 h). Where appropriate, food regime was switched at day 140 of the experiment. On day 205 of the experiment, half of the animals in each food regime were induced to metamorphose via immersion in 5-nM thyroxine solution. Each of the eight treatments was replicated seven times for a total of 56 animals.

Treatment code	Begun on	Switch	Thyroxine
Larvae			
HH	High food	No	No
HL	High food	Yes	No
LH	Low food	Yes	No
LL	Low food	No	No
Metamorphs			
HH	High food	No	Yes
HL	High food	Yes	Yes
LH	Low food	Yes	Yes
LL	Low food	No	Yes

TABLE 2

Summary of multivariate and univariate analyses of the effect of thyroid hormone (TH) treatment (thyroxine or T₄) and growth treatments on body mass (metamorphic mass for TH-treated salamanders and final mass for larval salamanders), gonad mass, and fat body mass. Mean squares for metamorphic mass are $\times 10^{-2}$, mean squares for larval period are $\times 10^{-7}$. For the multivariate statistics, $df = 3,39$ for TH; $df = 9,95$ for Growth; and $df = 9,95$ for TH $\times$ Growth. For univariate statistics, $df = 1,41$ for TH; $df = 3,41$ for Growth and TH $\times$ Growth. For body mass, error MS = 1.85×10^{-3} ; for gonad mass, error MS = 7.71×10^{-2} ; for fat body mass, error MS = 0.20.

Source	Multivariate statistics			Body mass			Gonad mass			Fat body mass		
	Wilks's λ	F	P	MS	F	P	MS	F	P	MS	F	P
TH	0.16	69.0	<0.001	0.349	188.89	<0.001	0.011	0.14	0.705	0.007	0.04	0.850
Growth	0.08	20.0	<0.001	0.186	100.50	<0.001	0.285	3.69	0.019	1.730	8.78	<0.001
TH $\times$ growth	0.80	1.0	0.444	0.003	1.98	0.132	0.039	0.51	0.675	0.006	0.03	0.993

TABLE 3

Summary of separate univariate analyses for larvae (i.e., salamanders not treated with T₄) and metamorphs (i.e., salamanders treated with T₄ to induce metamorphosis). ANOVAs tested the effect of growth treatments on larval period, body mass (metamorphic mass for T₄-treated salamanders and final mass for larval salamanders), gonad mass, and fat body mass. For larvae, df = 3,23; for metamorphs, df = 3,18.

	MS	F	P
Larvae			
Final mass	0.120	55.10	<0.001
Gonad mass	0.132	1.80	0.175
Fat body mass	0.958	6.40	0.003
Metamorphs			
Larval period	0.057	9.49	0.001
Metamorphic mass	0.076	53.70	<0.001
Gonad mass	0.175	2.12	0.133
Fat body mass	0.814	3.17	0.049

^aLarval period = time (d) to metamorphosis since beginning of thyroid hormone treatment.

TABLE 4

Summary of univariate analyses of the effect of sex on larval period (for thyroid hormone [TH] –treated salamanders), body mass (metamorphic mass for TH-treated salamanders and final mass for larval salamanders), gonad mass, and fat body mass. For the “all salamanders” analysis, $df = 1,47$; for the “metamorphs,” $df = 1,20$; for the “larvae,” $df = 1,25$.

	All salamanders			Metamorphs			Larvae		
	MS	F	P	MS	F	P	MS	F	P
Larval period	–	–	–	0.047	4.00	0.059	–	–	–
Body mass	0.003	0.14	0.709	0.004	0.36	0.557	0.026	1.66	0.209
Gonad mass	2.647	85.07	<0.001	1.595	78.02	<0.001	1.096	27.97	<0.001
Fat body mass	3.814	18.62	<0.001	1.277	4.40	0.049	2.621	17.74	<0.001

TABLE 5

Summary of analyses comparing female and male traits the in eight combinations of food (four treatments) and TH treatment (larvae vs. metamorphs). The value for “ratio” is the female mean divided by male mean. For metamorphs, “final mass” is mass at metamorphosis; for larvae, “final mass” is mass at termination. “Larval period” is number of days from initiation of TH-treatment until completion of metamorphosis. “LC” = constant low food; “LH” = low food switched to high food; “HL” = high food switched to low food; “HC” = constant high food.

	Larvae				Metamorphs			
	LL	LH	HL	HH	LL	LH	HL	HH
Larval period								
Ratio	–	–	–	–	1.20	1.37	1.29	1.16
df	–	–	–	–	1,4	1,2	1,5	1,3
MS	–	–	–	–	0.001	0.065	0.001	0.001
<i>F</i>	–	–	–	–	0.44	3.10	2.08	0.06
Final mass								
Ratio	0.88	0.83	0.88	1.06	0.93	1.04	1.03	0.93
df	1,5	1,5	1,5	1,4	1,4	1,2	1,5	1,3
MS	0.005	0.012	0.006	0.001	0.001	0.001	0.001	0.001
<i>F</i>	3.68	7.34	4.16	1.75	1.77	0.04	0.46	0.48
Gonad mass								
Ratio	2.08	5.54	2.85	2.68	4.19	3.37	2.88	2.76
df	1,5	1,5	1,5	1,4	1,4	1,2	1,5	1,3
MS	0.115	0.557	0.345	0.283	0.494	0.200	0.372	0.205
<i>F</i>	2.11	57.10 ^{**}	47.17 ^{**}	52.10 ^{**}	22.85 ^{**}	12.94	75.61 ^{***}	9.19
Fat body mass								
Ratio	0.51	0.21	0.16	0.34	0.57	0.21	0.25	0.31
df	1,5	1,5	1,5	1,4	1,4	1,2	1,5	1,3
MS	0.186	0.828	1.148	0.389	0.857	0.446	0.504	0.358
<i>F</i>	3.04	68.92 ^{***}	29.51 ^{**}	4.69	2.28	3.18	5.97	4.11

^{**} $P < 0.01$.

^{***} $P < 0.001$.